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Electron spin resonance studies of the interaction of oxidoreductases with 2,6-dimethoxy-*p*-quinone and semiquinone

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Previous electron spin resonance studies have demonstrated that the decay of ascorbyl plus semiquinone radicals, produced in an aqueous mixture of ascorbate and 2,6-dimethoxy-*p*-quinone, is accelerated by ascites cells. This effect was concluded to involve a sulfhydryl-containing NAD(P)H-enzyme, and work on cultured cell lines showed that on neoplastic transformation the activity against the radicals was increased. We show here that at least three disulfide-oxidoreductases are able to quench the radicals in a similar way to that of viable cells. Glutathione reductase (EC 1.6.4.2) in the presence of NADPH and oxidised glutathione, and dihydrolipoamide dehydrogenase (EC 1.8.1.4) with NADH and lipoamide, are found to accelerate the radical decay by reducing the quinone or semiquinone. DT-diaphorase (EC 1.6.99.2) in the presence of NAD(P)H can also achieve this by reducing the quinone directly. Lipoamide dehydrogenase and glutathione reductase are also capable of reducing nitroxide spin labels, a finding considered of relevance to the reported reduction of such spin labels by neuroblastoma cells.

Introduction

Free radicals can cause biological damage such as lipid peroxidation [1], mutagenicity [2,3] and protein damage [4], and some of the cellular defenses against such effects have been extensively studied [5]. Although spin traps have been employed for in vivo trapping of toxic free radicals [6], and spin labels have been commonly used for studying the physico-chemical properties of cell membranes [7], relatively few studies have investigated the ways in which viable cells directly interact with free radicals. We have reported [8,9] that Ehrlich ascites tumour cells are able to accelerate the rate of decay of an anoxic mixture of ascorbyl and semiquinone free radicals, and that for two cell culture systems the scavenging rate for transformed cells exceeds that of their non-transformed counterparts [10]. An effect similar to this last result was later reported [11] in studies of the reduction kinetics of spin labels incorporated into the membranes of differentiated and undifferentiated neuroblastoma cells.

We describe here the processes by which free radicals are generated and decay in ascorbate plus quinone mixtures, and the ways in which the kinetics of these processes can be modified by interactions with cells and enzymes. Previous stud-

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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ies [9] on subcellular fractions of ascites cells indicated that the scavenging of the ascorbyl plus semiquinone radicals is associated with an NAD(P)H-enzyme containing an active sulfhydryl group. We have now investigated how several oxidoreductases interact with the ascorbate plus quinone mixture and show here that at least three of them, namely glutathione reductase (EC 1.6.4.2), dihydrolipoamide dehydrogenase (diaphorase, EC 1.8.1.4) and DT-diaphorase (EC 1.6.99.2) are capable of accelerating the free radical decay kinetics in a way that is consistent with that observed in the cell studies. That other oxidoreductases may be capable of such radical scavenging is indicated by the finding [12,13] that thioredoxin reductase in skin can reduce spin-labelled quaternary ammonium salts.

Materials and Methods

Oxidized glutathione, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxy (3-carbamoyl-PROXYL) and 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy (TEMPOL) were obtained from Aldrich. The 2,6-dimethoxy-*p*-quinone was synthesized in the laboratory of Professor G. Fodor, University of West Virginia, and DT-diaphorase was the kind gift of Dr. Lars Ernster, Arrhenius Laboratory, Stockholm, Sweden. All other reagents were obtained from Sigma.

The method used to produce the ascorbyl and 2,6-dimethoxy-semiquinone radicals, as well the electron spin resonance (ESR) technique for monitoring the radical kinetics, was essentially the same as that described for the cell studies [8,9,14]. Two solutions in 50 mM Hepes buffer (pH 7.4) were prepared; the first contained 2 mM 2,6-dimethoxy-*p*-quinone, and the second 20 mM sodium ascorbate, plus 1 mg/ml superoxide dismutase and 5 mM ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA). At the beginning of each ESR experiment, 0.25 ml of each solution was added to 0.5 ml of the buffer and transferred by syringe into the aqueous flat cell of the ESR spectrometer. The modulation of the spectrometer was set at 4 gauss so that a signal representing a weighted combination of the ascorbyl and semiquinone resonances was obtained. This produced a good signal-to-noise ratio and permitted a fast spectrometer response time for the kinetic measurements. The ESR cell was flushed through with oxygen-free nitrogen before the start of each experiment, and this condition was maintained as the decay of the ESR signal was monitored for periods up to 1400 s after the initial mixing of the quinone and ascorbate solutions. The modifying effect of an enzyme on the decay kinetics of the radicals was determined by adding appropriate concentrations of the enzyme and of its substrates to 0.5 ml of the Hepes buffer immediately before the addition of the quinone and ascorbate solutions.

For studying the interaction of oxidoreductase enzymes with spin labels, 0.5 ml of Hepes buffer containing approximately 10 μM/l of TEMPOL or 3-carbamoyl-PROXYL was added at time zero to an equal volume of the buffer containing the test enzyme and substrate. The decay of the down-field resonance line of the spin label was monitored as a function of time until its magnitude had diminished by 90%. The entire resonance spectrum was then scanned to ensure that the loss of signal intensity did not reflect spectral changes resulting from immobilization of the spin label. Samples were maintained under a nitrogen atmosphere during the course of each experiment.

Results and Discussion

Previous studies [14] have shown that solutions of some quinones administered in combination with ascorbate were cytotoxic towards Ehrlich ascites tumours in mice. This synergistic cytotoxicity was found to be correlated with the production of long-lived populations of semiquinone and ascorbyl free radicals arising from one-electron reduction processes in the ascorbate plus quinone mixtures. The most toxic of the combinations tested was 2,6-dimethoxy-*p*-quinone (DMQ) with ascorbate, which produced a free radical concentration of about 4 μM/l with a half-life in excess of 10^3 s. The production of these radicals was found to be independent of oxygen and transition metals [15], and spin-trapping studies [15] indicated the absence of such short-lived intermediates as hydroxyl radicals.

The interaction of ascorbate with DMQ leads finally to the reduction of the quinone to its hydroquinone ($\mathrm{DMQH_2}$) and to the oxidation of ascorbate (A) to dehydroascorbate (DHA):

$$\mathrm{A + DMQ = DHA + DMQH_2} \quad (1)$$

This two-electron reduction proceeds through single-electron transfers and, as shown elsewhere [15], the two dominant steps that produce free radicals are:

$$\mathrm{DMQ + DMQH_2 = 2DM\dot{Q}} \quad (2)$$

and

$$\mathrm{2A + DMQ = 2\dot{A} + DMQH_2} \quad (3)$$

The rates of generation and decay of the ascorbate radical $\dot{\mathrm{A}}$ govern the rate of the overall reaction (1), whereas the concentration of the semiquinone radical $\mathrm{DM\dot{Q}}$ depends upon the extent of quinone reduction and reaches a maximum when the concentrations of DMQ and $\mathrm{DMQH_2}$ are equal in the reaction mixture. In the cell studies [8–10] and for the enzyme reactions reported here, it is the kinetics of these radicals that have been monitored. We have observed [9] that in accelerating the rate of decay of the radicals the cells consume NAD(P)H, so we can assume that the radical decay results from the complete reduction of one or more of the incompletely reduced species (i.e., A, DHA, DMQ or $\mathrm{DM\dot{Q}}$) in the above reactions. For our experimental condition of a 10-fold excess of the ascorbate over the quinone, it can be shown [15] that either a one- or two-electron reduction of the quinone, or a one-electron reduction of the semiquinone, has a more dominant effect on the overall radical decay kinetics than does a reduction of either dehydroascorbate or the ascorbyl radical. We show here that at least three oxidoreductases are capable of such reduction of the quinone or semiquinone.

The rate of decay of the semiquinone plus ascorbyl radicals in the buffer solution, together with the effect of adding various amounts of lipoamide dehydrogenase in the presence of 0.5 mM NADH, is shown in Fig. 1. No enzyme effect was observed if NADPH, rather than NADH, was used. The rate constants derived from the straight line plots of Fig. 1, together with those obtained from the same experiments using glutathione reductase in the presence of NADPH, are shown in Fig. 2 as a function of the enzyme concentration. Although the results for lipoamide dehydrogenase are consistent with its known native diaphorase activity [16], the concentrations of enzyme used (i.e., above 100 nM) were such that this activity is too small to have accounted for that observed in the cell studies [8–10].

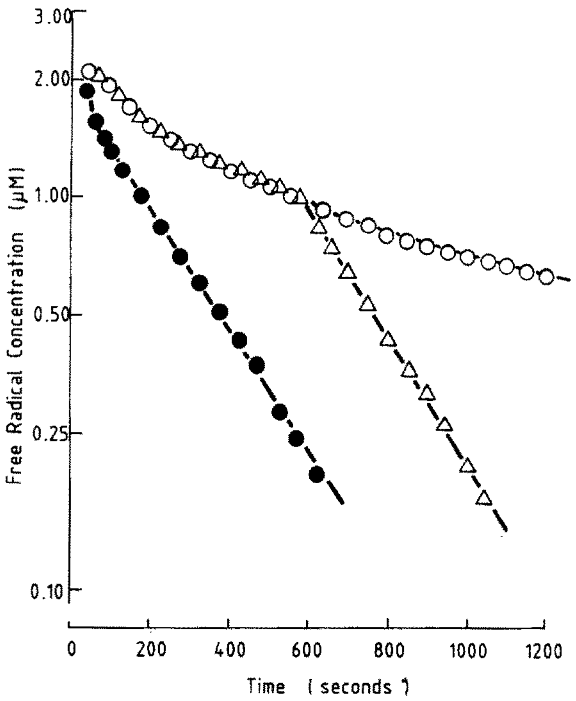

Fig. 1. The concentration of ascorbyl and semiquinone free radicals as a function of time after combining ascorbate and 2,6-dimethoxy-*p*-quinone in the presence of: (○) 0.5 mM NADH; () 0.5 mM NADH plus 139 nM lipoamide dehydrogenase; (△) 0.5 mM NADH, with 139 nM lipoamide dehydrogenase added after 600 s.

The activities of both lipoamide dehydrogenase and glutathione reductase were found to increase significantly in the presence of their appropriate disulfide substrates. The rate of decay of the radicals in the presence of 5.8 nM lipoamide dehydrogenase as a function of oxidized lipoamide and NADH concentrations is shown in Fig. 3 in the form of two Lineweaver-Burk plots. Cleland [17] has shown that parallel plots such as those of Fig. 3 are indicative of an enzyme operating through the so-called ping-pong mechanism in which re-

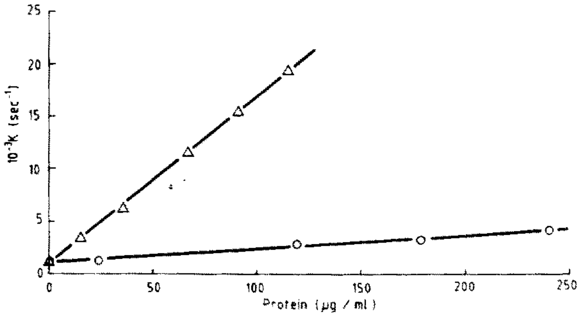

Fig. 2. The first-order rate constant k (determined from plots such as Fig. 1) as a function of lipoamide dehydrogenase and glutathione reductase concentrations in the absence of their disulfide substrates for: (△) lipoamide dehydrogenase plus 2 mM NADH; (○) glutathione reductase plus 2 mM NADPH.

lease of the first product precedes addition of the second substrate, with the enzyme existing in two stable forms. If negligible amounts of the products are present, the two-site ping-pong mechanism obeys the following rate equation [16]:

$$v = \frac{V}{1 + K_a/[A] + K_b/[B]} \tag{4}$$

where v and V are the initial and maximum velocities of the reaction, respectively. For lipoamide dehydrogenase the factors K_a and K_b can be taken as the Michaelis constants for lipoamide (A) and NADH (B), respectively. An analysis of the results of Fig. 3 in terms of Eqn. 4 leads to a Michaelis constant for lipoamide (corresponding to 0.15 mM NADH) of 0.45 mM and 35 ± 5 µM for NADH. Reed [18] obtained Michaelis constants for rat liver lipoamide dehydrogenase of 0.84 mM for lipoamide and 62 µM for NADH. For concentrations of NADH above 0.2 mM the lipoamide dehydrogenase activity decreased, implying an inhibitory action of NADH. Such substrate inhibition is consistent with a ping-pong enzyme mechanism [18,19]. Parallel Lineweaver-Burk plots were also obtained for glutathione reductase, and these provided a Michaelis constant of 48 ± 4 µM for oxidized glutathione, which can be compared with the value of 55 µM given for yeast glutathione reductase [20]. We observed that the quenching activity of glutathione reductase plus glutathione disulfide in the presence of NADPH fell off substantially with time, but that the full activity could be restored by the addition of more glutathione disulfide. This observation is compatible with either substrate depletion or with the formation of an adduct between reduced glutathione and 2,6-dimethoxy-*p*-quinone, which is a reaction well documented for other partially unsubstituted quinones [21].

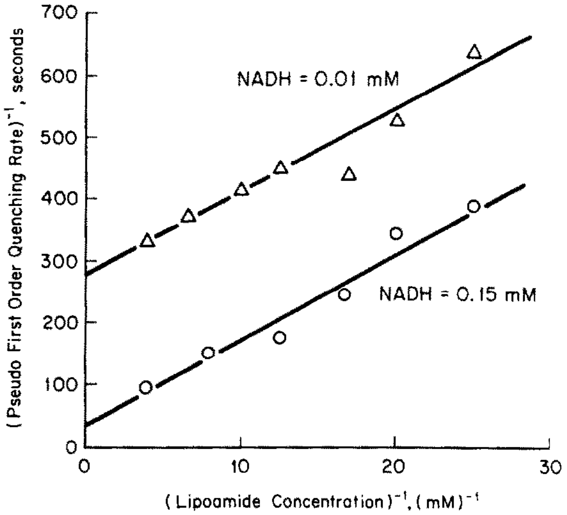

Fig. 3. Lineweaver-Burk plots [17] for 5.8 nM lipoamide dehydrogenase, using the rate constant k as a measure of the reaction velocity. △, 0.01 mM NADH; ○, 0.15 mM NADH.

DT-diaphorase is known to catalyze the two-electron reduction of quinones [22] using as the reducing source either NADH or NADPH with equal efficiency. Accordingly, this enzyme was found to accelerate the decay of the ascorbyl plus semiquinone radicals, and this activity is demonstrated in Fig. 4 for an NADH concentration of 2 mM. The activity of DT-diaphorase is increased by association with lipophilic agents and globular proteins [22] and, although this is not relevant for the results of Fig. 4 obtained on the pure enzyme, the diaphorase activity in the cells could have been increased by such influences.

The ability of lipoamide dehydrogenase to reduce the spin labels TEMPOL or 3-carbamoyl-PROXYL in the presence of 2 mM NADH, but without lipoamide, is shown in Figs. 5 and 6. Unlike the case shown in Fig. 1 for the activity of lipoamide dehydrogenase towards the ascorbate plus quinone mixture, the activity against the spin labels does not remain constant with time. This

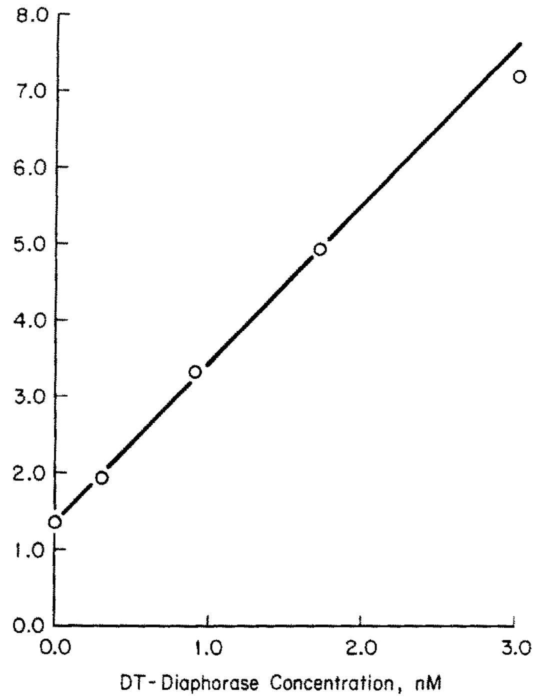

Fig. 4. First-order free radical decay rate as a function of enzyme concentration for DT-diaphorase in the presence of 2 mM NADH.

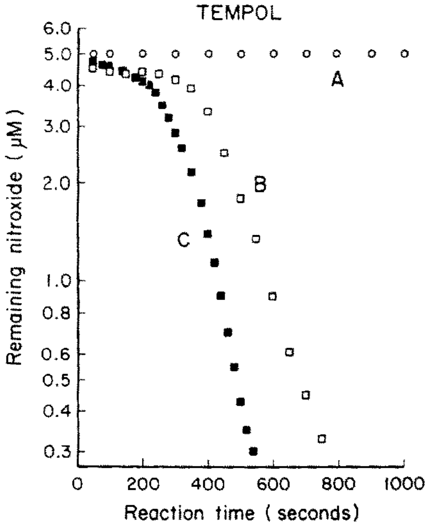

Fig. 5. The reduction of the spin label TEMPOL by (A) zero, (B) 6.5 units/ml, and (C) 12.5 units/ml of lipoamide dehydrogenase with 2 mM NADH, in the absence of lipoamide.

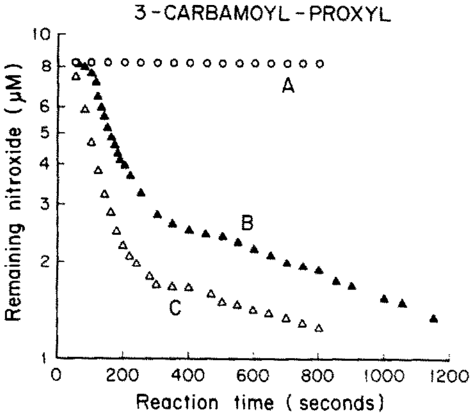

Fig. 6. The reduction of the spin label 3-carbamoyl-PROXYL by (A) zero, (B) 6.5 units/ml, and (C) 12.5 units/ml of lipoamide dehydrogenase with 2 mM NADH, in the absence of lipoamide.

suggests that the spin labels can influence the enzyme's activity, through denaturation or their acting as suicide substrates, for example. In the absence of NADH the enzyme was unable to reduce the spin labels. In the presence of 1 mM lipoamide the maximum rate of radical quenching was enhanced roughly 10-fold compared with that shown in Figs. 5 and 6, and first-order rate kinetics were observed. Glutathione reductase with NADPH was not capable of reducing the spin labels directly, but required the presence of oxidized glutathione. In their studies of the reduction of TEMPOL by neuroblastoma cells, Chen and McLaughlin [11] concluded that the cellular reductant was a non-protein-bound sulfhydryl group. Our results would suggest that a likely candidate for such a group could be lipoamide, or glutathione, or some other SH-based oxidoreductase substrate.

Conclusions

Our earlier studies [9] have shown that the elimination by ascites cells of the ascorbyl and semiquinone radicals, produced when ascorbate is mixed with 2,6-dimethoxy-*p*-quinone, is associated with an NAD(P)H-enzyme that contains an active sulfhydryl group. We have shown here that several sulfhydryl-oxidoreductases, namely lipoamide dehydrogenase, glutathione reductase and DT-diaphorase, are capable of modifying the decay kinetics of the ascorbyl plus semiquinone radicals in the same way as observed in the studies on ascites cells and cell culture systems [8–10]. This finding suggests that one or more of these oxidoreductase enzymes, or other related ones such as thioredoxin reductase, were responsible for the radical quenching observed in our earlier studies on viable cells and their homogenates. Future studies will attempt to clarify this.

Our previous ESR measurements were made on two families of cells capable of exhibiting either a normal or a transformed phenotype according to their culture conditions. The first consisted of normal rat kidney cells infected with a transformation virus that yielded a clone (6m2) exhibiting a transformed phenotype at 33°C and a normal phenotype at 39°C [10], and a clone of 6m2, designated 54-5A4, which was transformed at both 33°C and 39°C. The second consisted of chinese hamster ovary cells which exhibited a normal or transformed phenotype according to whether or not they were cultured in the presence of dibutyryl cyclic AMP. The ESR results indicated that in every case the radical quenching activity was significantly higher for the cells of transformed phenotype than for their normal counterparts. The results reported here suggest that this increased activity occurred because the transformed cells possessed one or more of the following features: a greater NAD(P)H-reducing power; a larger activity of disulfide oxidoreductases; a higher concentration of disulfide substrates such as glutathione or lipoamide. In this respect it is of interest to note that many tumour cells have been found to contain higher levels of DT-diaphorase than their normal counterparts [23]. The ability of lipoamide dehydrogenase to reduce spin labels suggests that changes in oxidoreductase activity accompanying neoplastic transformation may also account for the observation that neuroblastoma cells possess a greater capacity to reduce TEMPOL than do their normal homologs [11]. It will be of interest to investigate whether oxidoreductases play a more general role in protecting cells from free radical attack, and whether or not the enhancement of oxidoreductase activity on neoplastic transformation is a general effect.

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References

1 Tappel, A.L. (1980) in Free Radicals in Biology, Vol. 4 (Pryor, W.A., ed.), pp. 1–47, Academic Press, New York

2 Chesis, P.L., Levin, D.E., Smith, M.T., Ernster, L. and Ames, B.N. (1984) Proc. Natl. Acad. Sci. USA 81, 1696–1700

3 Morrison, H., Jernstrom, B., Nordenskjold, M., Thor, H. and Orrenius, S. (1984) Biochem. Pharmacol. 33, 1763–1769

4 Henriksen, T., Melo, T.B. and Saxebol, G. (1976) in Free Radicals in Biology, Vol. 2 (Pryor, W.A., ed.), pp. 213–256, Academic Press, New York

5 Bus, J.S. and Gibson, J.E. (1979) in Reviews in Biochemical Toxicology (Hodson, E., Bend, J.P., Philpot, R.M., eds.), pp. 125–149, Elsevier/North Holland, New York

6 Janzen, E.G. (1984) Methods Enzymol. 105, 188–198

7 Berliner, L.J. (1976, 1979) Spin Labeling. Theory and Applications, Vols. 1 and 2, Academic Press, New York

8 Pethig, R., Gascoyne, P.R.C., McLaughlin, J.A. and Szent-Györgyi, A. (1984) Proc. Natl. Acad. Sci. USA 81, 2088–2091

9 Pethig, R., Gascoyne, P.R.C., McLaughlin, J.A. and Szent-Györgyi, A. (1985) Proc. Natl. Acad. Sci. USA 82, 1439–1442

10 Gascoyne, P.R.C., McLaughlin, J.A., Pethig, R., Szent-Györgyi, A., Lai, C.-N. and Becker, F.F. (1984) Int. J. Quantum Chem. Quantum Biol. Symp. 11, 309–319

11 Chen, K.Y. and McLaughlin, M.G. (1985) Biochim. Biophys. Acta 845, 189–195

12 Schallreuter, K.U. and Wood, J.M. (1986) Biochem. Biophys. Res. Commun. 135, 221–227

13 Schallreuter, K.U. and Wood, J.M. (1986) Biochem. Biophys. Res. Commun. 136, 630–637

14 Pethig, R., Gascoyne, P.R.C., McLaughlin, J.A. and Szent-Györgyi, A. (1982) Proc. Natl. Acad. Sci. USA 80, 129–132

15 Gascoyne, P.R.C. (1986) Int. J. Quantum Chem. Quantum Biol. Symp. 13, in the press

16 Massey, V. (1958) Biochim. Biophys. Acta 30, 205–206

17 Cleland, W.W. (1967) Annu. Rev. Biochem. 36, 77–112

18 Reed, J.K. (1973) J. Biol. Chem. 248, 4834–4839

19 Garces, E. and Cleland, W.W. (1969) Biochemistry 8, 633–640

20 Serrano, A., Rivas, J. and Losada, M. (1984) J. Bacteriol. 158, 317–324

21 Irons, R.D. and Sawahata, T. (1985) in Bioactivation of foreign compounds (Anders, M.W., ed.), pp. 259–281, Academic Press, New York

22 Ernster, L. (1982) in Oxidases and related redox systems (King, T.T., Mason, H.S. and Morrison, M., eds.), pp. 321–347, Pergamon, Elmsford, New York

23 Smith, M.T. (1985) J. Toxicol. Environ. Health 16, 665–672